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Relative influences of DOC flux and subterranean fauna on microbial abundance and activity in aquifer sediments: new insights from ^{13}C-tracer experiments

ARNAUD FOULQUIER*, LAURENT SIMON*, FRANCK GILBERT†,‡, FRANCOIS FOUREL§, FLORIAN MALARD* AND FLORIAN MERMILLOD-BLONDIN*

*UMR CNRS 5023, Ecologie des Hydrosystèmes Fluviaux, Université Claude Bernard Lyon 1, Villeurbanne Cedex, France

†Université de Toulouse, UPS, INP, EcoLab (Laboratoire d'écologie Fonctionnelle), Toulouse, France

‡CNRS, EcoLab, Toulouse, France

§UMR CNRS 5125, PaléoEnvironnements & PaléobioSphère, Université Claude Bernard Lyon 1, Villeurbanne Cedex, France

SUMMARY

1. Aquifers are considered to be controlled bottom-up because of their dependence on organic matter supply from surface ecosystems. Microorganisms are generally assumed to form the base of the food web and to respond strongly to organic matter supply. Although the bottom-up control of microorganisms by carbon sources has been well documented, the potential top-down control of obligate groundwater invertebrates on microorganisms has never been addressed in alluvial aquifers.

2. The main aims of the present study were (i) to quantify the relative influences of the activity of a subterranean amphipod (*Niphargus rhenorhodanensis*) and the flux of dissolved organic carbon (DOC) on organic matter processing and microbial activity, biomass and abundance in slow filtration columns mimicking an alluvial aquifer, and (ii) to determine the feeding rate of *N. rhenorhodanensis* on sedimentary microbes by tracing the flux of a ^{13}C-labelled source of DOC in batches (closed systems).

3. Slow filtration column experiments showed that microbial abundance, biomass and activity were primarily controlled by DOC flux, whereas the activity of *N. rhenorhodanensis* had only a slight effect on the microbial compartment. Modelling of carbon fluxes in the ^{13}C-tracer experiments indicated that the feeding activity of the amphipod was too low to significantly modify microbial growth and activity.

4. Our experiments supported the hypothesis that groundwater ecosystems are controlled bottom-up. The small influence of *N. rhenorhodanensis* on the microbial compartment was probably linked to its slow metabolism. Our results highlight the need for further experiments to examine the relationship between metabolic rates of subterranean organisms and their role in ecosystem functioning.

Keywords: animal–microbe interactions, bottom-up control, carbon cycle, *Niphargus rhenorhodanensis*, top-down control

Correspondence: Florian Mermillod-Blondin, UMR CNRS 5023, Ecologie des Hydrosystèmes Fluviaux, Université Claude Bernard Lyon 1, Bât Forel, 43 Bd 11 Novembre 1918, F-69622 Villeurbanne Cedex, France.
E-mail: mermillo@univ-lyon1.fr

Introduction

Aquatic subterranean ecosystems are heterotrophic, their functioning depending on the supply of allochthonous organic matter originating from surface ecosystems. The lack of photosynthesis (and primary production) in ground water implies that the food

webs are truncated (Gibert & Deharveng, 2002). Theory predicts that heterotrophic and organic matter-limited environments such as groundwater ecosystems are controlled bottom-up because the quantity and quality of organic matter entering the system determine food web structure (Malard *et al*., 1994; Notenboom, Plénet & Turquin, 1994; Datry, Malard & Gibert, 2005). The soil and vadose zone (between the ground surface and the water table) act as strong physical, chemical and biological filters that dramatically reduce the transfer of organic detritus (particulate organic matter – POM) from surface to ground water. Consequently, dissolved organic carbon (DOC) is the main source of food for life in aquifers (e.g. Starr & Gillham, 1993). Accordingly, Simon, Benfield & Macko (2003) showed that microorganisms which process DOC represented the main food resource for higher trophic levels in a karstic system. In the hyporheic zone, several studies (e.g. Findlay *et al*., 1993; Claret & Fontvieille, 1997; Craft, Stanford & Pusch, 2002) have demonstrated the significance of DOC availability for microbial growth, with microorganisms being a potential food source for invertebrates (Bärlocher & Murdoch, 1989). In a phreatic aquifer, Datry *et al*. (2005) reported increased densities of groundwater invertebrates in zones enriched with DOC and suggested that a DOC-induced enrichment of microbial biomass stimulated the abundances of groundwater invertebrates.

Although this 'bottom-up view' of groundwater ecosystems is largely accepted, there have been no attempts to quantify the influence of the obligate groundwater fauna on the microbial compartment and DOC consumption and assimilation in alluvial groundwater ecosystems (Gibert *et al*., 2009). In a recent review, Boulton *et al*. (2008) pointed out that obligate groundwater invertebrates should affect many of the microbial processes involved in the C and N cycles in a similar way to that observed for surface-dwelling aquatic invertebrates. In sediments, grazing activities of invertebrates may enhance biofilm productivity (e.g. Traunspurger, Bergtold & Goedkoop, 1997), whereas bioturbation activities (burrowing, gallery building, and faecal pellet production) may indirectly influence microbial communities through modifications of physicochemical conditions (Danielopol, 1989; Mermillod-Blondin & Rosenberg, 2006). However, clear evidence of the role of invertebrates in microbial processes in sediment has essentially been limited to surface-dwelling species that lack many of the behavioural and physiological adaptations to food limitation shared by most obligate groundwater invertebrates (Hervant, Mathieu & Barré, 1999a). Thus, the main aim of the present study was to test whether a model obligate groundwater species, the amphipod *Niphargus rhenorhodanensis* Schellenberg had a significant influence on the microbial compartment and organic matter processing in aquifer sediments. This species was selected because it is the most abundant and widely distributed subterranean macroinvertebrate in the alluvial aquifer of the Rhône River (Dole-Olivier & Marmonier, 1992). Two laboratory experiments were developed (i) to quantify the relative influences of amphipod activities (top-down control) and the flux of DOC (bottom-up control) on the microbial compartment and DOC processing in slow filtration columns mimicking an alluvial aquifer habitat and (ii) to determine the feeding rate of *N. rhenorhodanensis* on attached bacteria by tracing the flux of a ^{13}C-labelled source of DOC in batches.

Methods

Collection of sediments

Gravel collected from the Rhône River was sieved manually to select particle sizes ranging from 5 to 8 mm and then was cleaned with deionised water before being dried at 60 °C. For the two experiments, 10 kg of fresh sand was collected at a depth of 1 m below the bed of a gravel pit intersecting the water table of a glaciofluvial aquifer using the Bou–Rouch method (Bou & Rouch, 1967). DOC concentrations in ground water collected from this glaciofluvial aquifer ranged from 0.4 to 0.9 mg L^{-1}. Groundwater temperature was 14.5 ± 0.3 °C, and dissolved oxygen (DO) concentrations ranged from 5.8 to 6.7 mg L^{-1}. Particulate organic carbon contained in the collected sand was 0.42 ± 0.03 mg g^{-1} of sediment dry mass, and the abundance of bacteria associated with sand was 2.1 10^8 bacteria g^{-1} of sediment dry mass. After collection, the sand was manually sieved to select particle sizes ranging from 100–1000 μm and then kept in an oxygenated water bath in the laboratory two days before experiments started.

Collection of subterranean organisms

The obligate groundwater amphipod *Niphargus rhenorhodanensis* (dry weight = 1.0–1.3 mg), which inhabits both karstic and alluvial aquifers, is an opportunistic species showing a generalist feeding strategy (Ginet, 1960; Danielopol, 1989). Individuals of *N. rhenorhodanensis* were collected using traps buried at a depth of 5 cm in the sediment of a ditch draining the alluvial aquifer of the Dombes Forest, France (45°58′28′N, 5°24′25′E, clade I in Lefébure *et al.*, 2007). To acclimatise animals to experimental conditions (temperature, granulometry and food), they were maintained in the laboratory for more than 15 days before the experiment started.

Experiment I: relative influences of N. rhenorhodanensis and DOC flux on microbial compartment in slow filtration microcosms

Slow filtration column experiments were conducted to test for the influences of amphipod activity (top-down control) and the flux of DOC (bottom-up control) on microbial compartment and DOC processing in sediments. Columns had an inside diameter of 10 cm and consisted of two experimental modules (10 cm high) topped by a third module of 5 cm in height (Fig. 1a). They were filled with inert gravel and fresh sand to a height of 15 cm. About 10 cm of water was left above the sediment surface. Masses of gravel (500, 500 and 400 g) and sand (150, 150 and 120 g) were alternately introduced into the columns to obtain a heterogeneous porous media. During sediment introduction, particle tracers (luminophores) were deposited at depths of 2.5–3.5 cm (1 g of yellow luminophores) and 6–7 cm (1 g of pink luminophores) below the sediment surface to estimate sediment reworking induced by amphipods (method described below).

Experiments were performed at constant temperature (15 ± 0.5 °C) in the dark. The columns were supplied from the top with reconstituted ground water (96 mg L^{-1} $NaHCO_3$, 39.4 mg L^{-1} $CaSO_4$ $2H_2O$, 60 mg L^{-1} $MgSO_4$ $7H_2O$ and 4 mg L^{-1} KCl, 19 mg L^{-1} $Ca(NO_3)_2$ $4H_2O$) using a peristaltic pump controlling a constant infiltration flow rate of 2 mL min^{-1}. Supplied water was aerated to maintain concentrations of DO between 8.5 and 9.5 mg L^{-1} at the inlet of the columns. The experiment was designed to couple three modalities of DOC flux (i.e. 60, 180 and 300 μg h^{-1} of C by modifying the concentration of easily degradable DOC sodium acetate supplied to columns) with two modalities of fauna (absence or

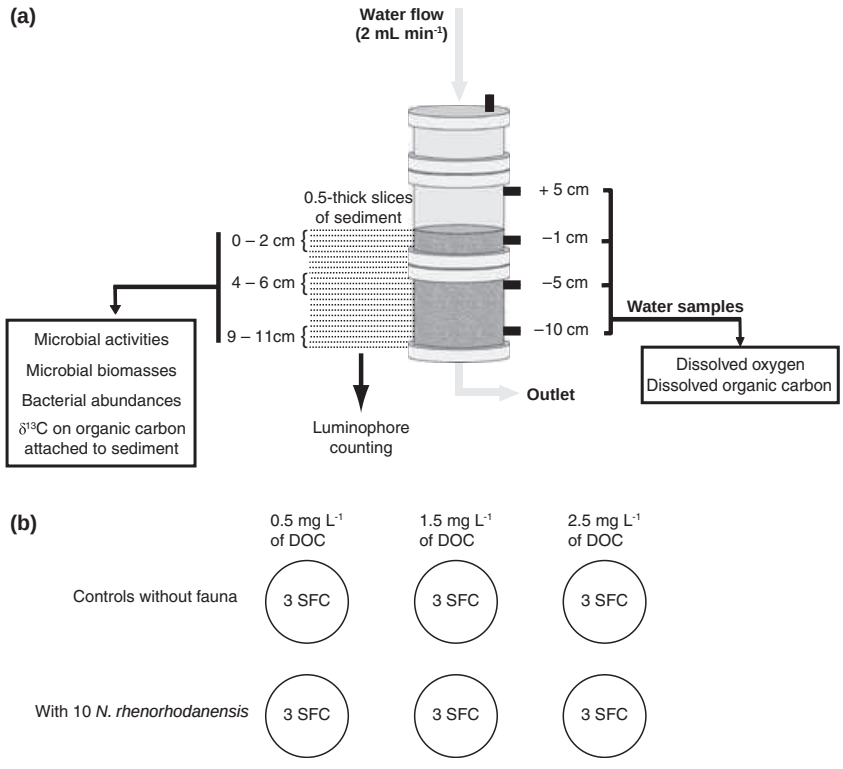

Fig. 1 Schematic representation of slow filtration columns indicating the positions of sampling (a) and experimental design of the experiment I in slow filtration columns (b). SFC = slow filtration column.

presence of 10 individuals of *N. rhenorhodanensis*) to test for the interaction between DOC supply and the activity of *N. rhenorhodanensis* on organic matter processing and microbial parameters (Fig. 1b). A total of six experimental treatments were tested with three replicate columns per treatment. Dissolved acetate supplied to all columns was enriched with 0.2% of ^{13}C-marked acetate ($^{13}C_2H_4O_2$, 99 atom% ^{13}C; Sigma–Aldrich, Saint-Quentin Fallavier, France) to determine the assimilation of DOC in attached bacteria and amphipods. The δ^{13}C of the DOC solution supplied to columns was 135 ‰.

After 1 week of column stabilisation, 10 individuals of *N. rhenorhodanensis* were introduced in half of the columns (n = 3 columns per DOC treatment). Amphipod density in slow filtration columns (i.e. 8.4 individuals L^{-1} of sediment) was similar to that measured in artificial substrata inserted in the bed sediment of streams (7.2 ± 2.6 individuals L^{-1} of sediment, Mathieu & Essafi-Chergui, 1990). Measurements of DOC and DO were made at four depths (5 cm above the sediment surface, and 1, 5 and 10 cm below the sediment surface) on days 0 (before addition of amphipods in the system), 6, 10, 14, 18, 22 and 26 after the addition of *N. rhenorhodanensis* to determine the removal rates of DOC and DO (aerobic respiration) in slow filtration columns (Fig. 1a). After the last measurements (day 26), columns were dismantled, the water layer was removed and columns were opened to sample sediment (Fig. 1a). The top 11 cm of fresh sediment were sampled in 0.5 cm thick slices. Each layer was sieved to remove gravel and invertebrates, homogenised, and a 1 g sub-sample was used for sediment reworking analyses (see below). Remaining fresh sediments corresponding to three layers (0–2, 4–6 and 9–11 cm below the sediment surface) were used to analyse the $^{13}C/^{12}C$ ratio of bacteria (incorporation of acetate in bacteria, see model in Data analysis section), bacterial abundance (numbers of bacteria and active eubacteria), microbial activities (hydrolytic activity, dehydrogenase activity) and biochemical markers of biofilm biomass (carbohydrates and proteins). Carbohydrates and proteins were used to estimate biomasses of extracellular polymeric substances and bacterial cells, respectively (Foulquier A., Mermillod-Blondin F., Malard F. & Gibert J., *submitted*). Individuals of *N. rhenorhodanensis* were recovered from columns to analyse their $^{13}C/^{12}C$ ratio and calculate the incorporation rate of ^{13}C-enriched microbial biomass in animals (see model in Data analysis section). After collection, *N. rhenorhodensis* were kept 48 h in glass bowls with tap water to clear their gut contents before stable isotope analyses.

Experiment II: incorporation of DOC in the food web

We designed a second experiment with simplified experimental units to quantify the assimilation of DOC in attached bacteria and subterranean amphipods. A total of 36 erlenmeyer flasks were used to measure bacterial growth and the temporal dynamics of $^{13}C/^{12}C$ ratio in bacteria and amphipods. Flasks (250 mL) were filled with 30 g dry mass of fresh sand (collected and treated as in Experiment I) and 160 mL of reconstituted ground water (ionic composition as in Experiment I). Reconstituted ground water was enriched with ^{13}C-labelled acetate ($^{13}C_2H_4O_2$, 99 atom% ^{13}C) to fit an initial concentration of 25 mg L^{-1} of DOC and a $^{13}C/^{12}C$ ratio of 47 atom%. The concentration of DOC (25 mg L^{-1}) used in this experiment was higher than the DOC concentrations used in slow filtration columns (<3 mg L^{-1}). However, the application of a high concentration of ^{13}C-labelled DOC was necessary to obtain significant ^{13}C enrichments in bacteria and amphipods for the calculation of carbon assimilation rates. Four individuals of *N. rhenorhodanensis* were introduced in half of the flasks (n = 18) to measure their capacity to modify DOC incorporation in attached bacteria and to feed and incorporate bacterial carbon in their tissues. At six dates during the experiment (0, 12, 24, 48, 72 and 96 h), three flasks of each treatment (absence or presence of *N. rhenorhodanensis*) were killed to determine DOC concentrations in the overlying water, number of bacteria and active eubacteria on the sediment, and the $^{13}C/^{12}C$ ratios of the bacteria and amphipods (used for calculation of assimilation rates, see model in Data analysis section). As indicated for experiment I, collected amphipods were kept 48 h in glass bowls with tap water to clear their gut contents.

Physical, chemical and microbial analyses

Sediment reworking analyses. Sediment reworking by amphipods was assessed using luminophores (natural sediment particles of 350–500 μm in size, dyed with a fluorescent paint) as particle tracers (Gerino *et al.*, 1998). Each sub-sample of fresh sediment

collected at the end of the experiment from 0.5 cm thick slices was lyophilised for luminophore counting. Detection of luminophores was performed under UV light (digital camera Olympus C-2500L; Olympus France, Rungis, France) for automatised counting on acquired images (image analysis software Image-Pro Plus). Luminophore quantities were reported in terms of quantity of dry sand collected in each slice.

Dissolved oxygen and DOC analyses. Dissolved oxygen measurements in slow filtration columns were made with an oxygen microsensor probe (Unisense, Aarhus, Denmark) directly connected to the four lateral water outlets, thereby preventing any contact with atmospheric oxygen. Water samples for DOC analyses were filtered through HAWP filters (porosity: 0.45 μm; Millipore, Billerica, MA, U.S.A.) and analysed with a total carbon analyser (multi N/C® 3100; Analytik Jena, Jena, Germany) based on thermocatalytic oxidation and MC-NDIR detection after removing inorganic carbon with hydrochloric acid (5 μL mL^{-1}) and CO_2 stripping under 15 min oxygen flow.

Stable isotope analysis. Sediment samples (with attached bacteria) and amphipods were oven dried at 50 °C for at least 48 h and then crushed using a mortar and pestle. About 500 mg of dry sediments were placed in pre-cleaned Oakridge centrifugation tubes and 2 M HCl was added for 12 h at room temperature to remove calcite. After centrifugation at 2500 *g* during 5 min, the supernatant was discarded; sediments were rinsed three times with ultrapure water and oven dried at 50 °C. An amount of 2.5 mg of dry sediments were weighted in tin capsules for stable isotope analysis. Dry amphipods were placed in glass vials and acidified with 400 μL of 2 M HCl to remove calcium carbonate. Samples were oven dried at 50 °C without subsequent rinse. A total of 250 μg of dry amphipod were weighted in tin capsules for stable isotope analysis.

Stable isotope ratios of carbon ($^{13}C/^{12}C$) were measured by continuous flow stable isotope ratio mass spectrometer (CF-IRMS) using a GVI Isoprime mass spectrometer interfaced with a Eurovector Euro-EA3028-HT elemental analyser. $^{13}C/^{12}C$ ratios were expressed as δ in part per thousand (‰) and referenced to V-PDB standard. The analytical precision achieved for tyrosine standards analysed along with the samples was better than 0.1‰ (± standard deviation).

Microbial analyses. The DNA intercalating dye (DAPI) and a Cy3 probe (EUB 338, eubacteria) were used on sediment samples to determine the total number of bacteria and the percentages of active eubacteria. Sediment samples (1 g) were fixed, homogenised, spotted on slides and hybridised according to Mermillod-Blondin *et al.* (2005). Slides were mounted with Citifluor solution (Citifluor Ltd, London, U.K.), and the preparations were examined at 1000× magnification with a BH2-RFCA Olympus microscope fitted for epifluorescence with a high-pressure mercury bulb (50 W) and filter sets BP 405 (for DAPI) and BP 545 (for Cy3). Bacteria from the samples were analysed in 20 fields per sample with up to 30 cells per field. Numbers of DAPI and Cy3 bacteria were counted separately from the same field to determine the percentages of active eubacteria (% Cy3 bacteria/DAPI bacteria). Total numbers of bacteria were expressed as number of cells g^{-1} dry weight (DW) of sediment.

The 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl tetrazolium chloride (INT) was used to measure dehydrogenase activity as modified from Houri-Davignon, Relexans and Etcheher (1989). Sediment samples (1 g fresh weight) were incubated into a 0.02% INT solution (final solution) for 2 h at 15 °C and then filtered on a nylon membrane (0.22 μm, MSI). Controls were prepared by adding formaldehyde (2% final) in INT solution. Extraction of INT formazan was made in vials containing 5 mL of methanol. Each vial was sonicated at 100 W during two periods of 60 s using a sonicator fitted with a 2-mm-diameter probe (Sonicator XL 2020; Misonix Inc., Farmingale, NY, U.S.A.) to increase solvent extraction yield (Maurines-Carboneill *et al.*, 1998). The INT formazan extract was measured by a spectrophotometer adjusted at 480 nm against control blank. The quantity of INT formazan was computed by using the molar extinction coefficient of 18 000 M^{-1} cm^{-1} at 480 nm and was expressed as μmol of INT h^{-1} g^{-1} DW of sediment.

Hydrolytic activity was estimated using the fluorescein diacetate (FDA) hydrolysis method (Jørgensen, Eriksen & Jensen, 1992). Sediment samples (1 g fresh weight) were placed into 3 mL of a pH 7.6 phosphate buffer solution with 0.15 mL of 4.8 mM FDA solution. The incubation was maintained for 1 to 3 h until a green colouration of fluorescein appeared. The reaction was stopped by freezing the sample after the addition of 4.5 mL of a solution of $HgCl_2$

(400 mg L^{-1}). Fluorescein concentration was estimated from the absorbance of the filtered supernatant (0.45 μm, Millipore) measured at 490 nm and was expressed as μmol of FDA h^{-1} g^{-1} DW of sediment.

Total proteins on sediment were measured according to the Lowry method modified by Peterson (1977), using the Sigma Protein Assay Kit (P 5656 Sigma Diagnostics, St Louis, MO, USA). Concentration was expressed as mg of protein g^{-1} DW of sediment.

Carbohydrates were quantified on dry sediment (24 h at 60 °C in an oven) using the Dubois method (Dubois *et al*., 1956). A homogenised sample of dry sediment (0.25 g) was mixed with 1 mL of a phenol solution (10%) and 5 mL of sulphuric acid (95%). Incubation was performed at room temperature in the dark for 1 h. Absorbance of the supernatant was measured at 495 nm, and carbohydrate content was expressed as mg of carbohydrates g^{-1} DW of sediment.

Data analysis

Experiment I. The quantification of luminophore redistribution following sediment reworking by *N. rhenorhodanensis* was realised using a model inspired by the gallery-diffusion model of François *et al.* (2002). This model describes both the diffusive-like mixing of particles in the region of intense burrowing activity (D_b) and the non-local mixing pattern (r) associated with a biologically induced transfer of particles from one layer to another in a discontinuous pattern (i.e. a non-continuous transport; Boudreau, 1986; Meysman, Boudreau & Middelburg, 2003). Detailed information about this model can be found in Gilbert *et al.* (2007). Biodiffusion-like coefficients of particles obtained for yellow and rose luminophores were compared among DOC and fauna treatments using a two-way analysis of variance (ANOVA). Tukey's *post hoc* tests were performed to identify significant pairwise differences between treatments.

Concentrations of DO and DOC measured on day 26 at 4 depths in slow filtration columns were used to calculate the removal rate of DOC and DO in three sediment layers (i.e. 0–2, 2–5 and 5–10 cm below the sediment surface) as follows:

$$RR = (\Delta_C \times Q)/(V \times P) \quad (1)$$

Where:

RR: Removal rate of DOC and DO in mg h^{-1} L^{-1} sediment

Δ_C: Difference in DOC or DO concentration (mg L^{-1}) between two consecutive layers

Q: water discharge (L h^{-1}) through the sediment layer

V: volume of the sediment layer (L)

P: porosity

A three-way ANOVA was used to test for the effects of depth, DOC flux and amphipods on the removal rates of DO and DOC, microbial parameters (abundance, activities and biochemical markers) and $^{13}C/^{12}C$ ratio of attached bacteria. A one-way ANOVA was used to compare the $^{13}C/^{12}C$ ratio of amphipod tissues collected at the end of the experiment between DOC treatments. Tukey's *post hoc* tests were performed to identify significant pairwise differences between treatments.

Experiment II. Student's *t*-tests were performed on measurements made at 0 h to verify that there were no differences in DOC concentrations, bacterial counts and $^{13}C/^{12}C$ ratios of attached bacteria between the 2 series of flasks (n = 3) attributed to each fauna treatment before addition of *N. rhenorhodanensis*. After 0 h, a two-way ANOVA with fauna (presence or absence of *N. rhenorhodanensis*) and time (12, 24, 48, 72 and 96 h) was used to test for differences in the temporal dynamics of DOC, bacterial counts and $^{13}C/^{12}C$ ratios of attached bacteria between fauna treatments. A one-way ANOVA was used to compare the $^{13}C/^{12}C$ ratios of amphipod tissues among dates.

Modelling of carbon fluxes. The modelling of carbon fluxes between DOC, attached bacteria and *N. rhenorhodanensis* was performed using a similar approach as in Mauclaire *et al.* (2003). Bacteria growth was assumed to follow Monod kinetics (Monod, 1942). The bacterial uptake of DOC (C_{DOC}) over time t in the experimental system was described by the following differential equation:

$$\frac{dC_{DOC}}{dt} = -\frac{1}{Y_1}\mu_1 \frac{C_{DOC}}{K_s + C_{DOC}} B \quad (2)$$

where B was the carbon biomass of bacteria (g C bacteria), K_S was the Monod constant (mg C), μ_1 was the maximum growth rate of bacteria (day^{-1}) and Y_1 was the carbon conversion efficiency between acetate and bacterial biomass (g C bacteria g^{-1} C_{DOC}). The

feeding rate of amphipods on bacteria was assumed to depend on the bacterial biomass and we neglected biomass changes because of natural decay, which yielded the equation:

$$\frac{dN}{dt} = \mu_2 BN \tag{3}$$

with N as amphipod biomass (g C amphipods) and μ_2 as growth rate (g^{-1} C bacteria day^{-1}).

Total change of bacteria biomass was then calculated as follows:

$$\frac{dB}{dt} = \left(\mu_1 \frac{C_{DOC}}{K_s + C_{DOC}} B\right) - \mu_3 B - \frac{1}{Y_2}\mu_2 BN \tag{4}$$

where μ_3 was the bacteria natural decay rate (day^{-1}) and Y_2 was the carbon conversion efficiency between bacteria and amphipod biomasses (g C amphipods g^{-1} C bacteria).

^{13}C fluxes were modelled to describe changes in the $^{13}C/^{12}C$ ratio of attached bacteria and amphipod tissues. We assumed that there was no isotopic fractionation between DOC and attached bacteria, and between bacteria and amphipods during C assimilation. This assumption was a reasonable simplification because C isotope fractionations during aerobic metabolism are generally low (0 to few ‰), thus negligible relatively to the high ^{13}C enrichment of this study. The change of ^{13}C mass in DOC was given by:

$$\frac{d^{13}C_{DOC}}{dt} = -\frac{1}{Y_1}\mu_1 \frac{^{13}C_{DOC}}{K_s + C_{DOC}} B \tag{5}$$

while the evolution through time of ^{13}C in bacteria ($^{13}C_B$) and amphipods ($^{13}C_N$) was expressed as:

$$\frac{d^{13}C_B}{dt} = \left(\mu_1 \frac{^{13}C_{DOC}}{K_s + C_{DOC}} B\right) - \mu_3\, ^{13}C_B - \frac{1}{Y_2}\mu_2\, ^{13}C_B N \tag{6}$$

$$\frac{d^{13}C_N}{dt} = \mu_2\, ^{13}C_B N \tag{7}$$

The carbon mass and the isotopic compositions of the DOC, bacteria and amphipods were computed at each time step by solving the system of differential equations (2–7) that described the carbon and ^{13}C mass balances using a fourth-order Runge-Kutta numerical integration procedure (Press *et al*., 1992). To compare the result of the model to the experimental data, the carbon isotope composition of total organic carbon attached to sediment (comprising the organic carbon contained in bacterial cells and bacterial exopolymeric substances) was calculated using a simple mass-balance equation:

$$\delta^{13}C_{TOC} = \frac{B\delta^{13}C_B + C^i_{TOC}\delta^{13}C^i_{TOC}}{B + C^i_{TOC}} \tag{8}$$

where C^i_{TOC} was the total organic carbon attached to sediments at the start of the experiment.

Cell bacterial biomass at the start of the experiment was estimated from DAPI counts using a conversion factor of 30 fg of C $cell^{-1}$ (Fukuda *et al*., 1998; Troussellier *et al*., 1997). Invertebrates contained about 500 μg of C per individual at $t = 0$.

Unknown parameters of the model (μ_1, μ_2, μ_3, K_S, Y_1 and Y_2, Table 1) were calculated by adjusting the simulation results to measured DOC concentrations, $\delta^{13}C$ values of total organic carbon and $\delta^{13}C$ of amphipod tissues. The quality of the fit between modelled and measured data was evaluated by calculating the sum of the squared differences between observed and calculated values.

For all parametric tests, homoscedasticity and data normality were checked using Levene's tests and Shapiro's tests, respectively. Whenever necessary, data were ln or square-root transformed to homogenise variances and fit data normality. For variables

Table 1 Biodiffusion-like coefficients (Db) calculated for luminophores deposited in two depth layers of the columns for the six experimental treatments. DOC1 = 60 μg C h^{-1}, DOC2 = 180 μg C h^{-1}, and DOC3 = 300 μg C h^{-1}. Values are means (SD). n = 3 columns per treatment

Depth of luminophore deposition (cm)	Treatment	Db (cm^2 $year^{-1}$)
2.5–3.5	DOC1 * control	0.76 (0.39)
	DOC1 * *N. renorhodanensis*	0.52 (0.32)
	DOC2 * control	0.36 (0.17)
	DOC2 * *N. renorhodanensis*	0.47 (0.40)
	DOC3 * control	0.15 (0.02)
	DOC3 * *N. renorhodanensis*	0.17 (0.05)
6.0–7.0	DOC1 * control	0.85 (0.19)
	DOC1 * *N. renorhodanensis*	1.07 (0.46)
	DOC2 * control	1.29 (0.66)
	DOC2 * *N. renorhodanensis*	0.84 (0.28)
	DOC3 * control	1.09 (0.76)
	DOC3 * *N. renorhodanensis*	0.84 (0.14)

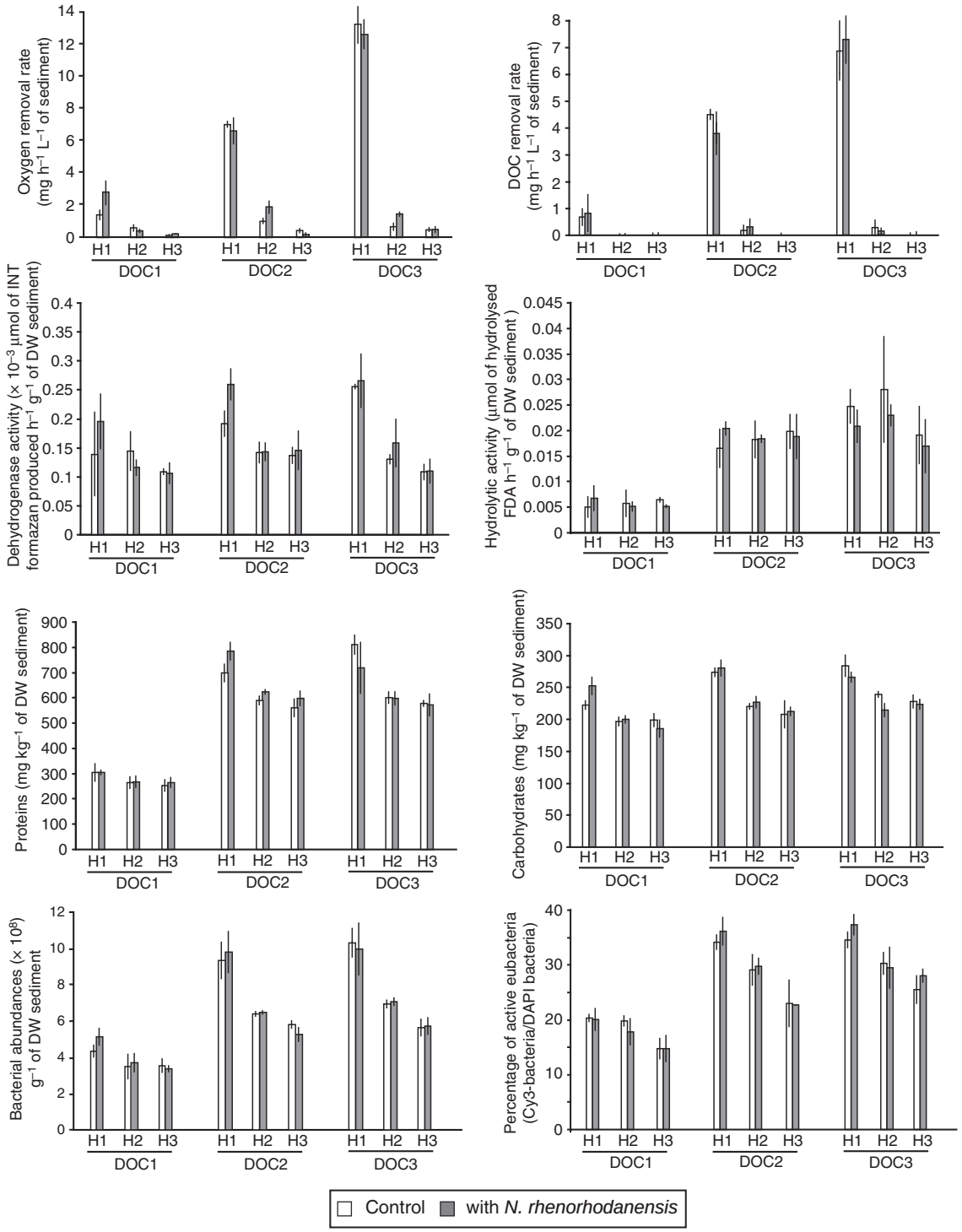

Fig. 2 DO and DOC removal rates, and microbial activities, biomasses and abundances measured at three layers in columns for the six tested treatments (3 DOC flux treatment × 2 animal treatments) in experiment I. Symbols for DOC supply: DOC1 = 60 μg C h^{-1}, DOC2 = 180 μg C h^{-1} and DOC3 = 300 μg C h^{-1}. Symbols for depth layers: H1 = 0–2 cm, H2 = 4–6 cm and H3 = 9–11 cm below the sediment surface. Values are means ± SD (n = 3 columns per treatment).

expressed as percentages (% of active eubacteria), data were arcsine transformed. All statistical analyses were performed using Statistica 5™ (Statsoft, Tulsa, OK, U.S.A.).

Results

Experiment I: relative influences of N. rhenorhodanensis and DOC flux on microbial compartment in slow filtration microcosms

The luminophore vertical profiles were diffusive-like shapes, indicating a preferential occurrence of random mixing events over short distances in the columns. This induced the production of diffusion-like coefficients (D_b) and the absence of non-local coefficients (r) by the gallery-diffusor model. The diffusion-like coefficients (Db) were not significantly affected by the presence and activity of the amphipods (Table 1, two-way ANOVA, fauna effect, $F_{(1.12)} < 0.4$, $P > 0.5$ for the two investigated sediment layers).

Most variables showed no significant influence of *N. rhenorhodanensis* on the microbial compartment (Fig. 2, Table 2). Amphipods had only significant positive effects on DO removal rate and dehydrogenase activity in slow filtration columns, but these effects depended on depth as well as on 'depth × DOC flux' interactions for DO removal rate (Table 2). For instance, *Niphargus rhenorhodanensis* increased DO removal rate by 97% in the top 2 cm of sediment columns receiving a DOC flux of 60 μg C h^{-1}, whereas it had no significant effect (−5.4%) at the same depth in the treatment with a DOC flux of 180 μg h^{-1} (Fig. 2).

On the contrary, the increase in DOC flux had a significant positive effect (Table 2, Fig. 2) on microbial processes (DO removal, DOC removal, and dehydrogenase and hydrolytic activities), microbial biomass (proteins and carbohydrates) and bacterial counts (total number of cells and percentage of active eubacteria). In the top 2 cm of sediments, the DOC removal rate, DO consumption, protein concentration and bacterial abundance increased by 552, 389, 129 and 102%, respectively, as the DOC flux increased from 60 to 180 μg C h^{-1}. The influence of DOC supply on microbial parameters decreased with increasing depth in slow filtration columns except for hydrolytic activity (Table 2). As a consequence of the influence of vertical DOC fluxes on microorganisms, all microbial variables were positively correlated with the DOC removal rates measured in slow filtration columns (Table 3).

The δ^{13}C value of total organic carbon attached to sediment also increased significantly with increasing DOC flux and decreased with depth (Fig. 3a, Table 2). This δ^{13}C signature was highly correlated with the DOC removal rate and the microbial variables (Table 3) indicating that carbon from acetate was incorporated into bacterial biomass. *N. rhenorhodanensis* did not have a significant influence on δ^{13}C of total organic carbon attached to sediment (Table 2) but δ^{13}C values of amphipods showed a higher incorporation of organic carbon in their tissues for the highest DOC treatment (Fig. 3b, one-way ANOVA, $F_{(3,9)} = 7.4$, $P < 0.01$).

Experiment II: incorporation of DOC in the food web

DOC decreased rapidly with time (Fig. 4a, Table 4) owing to consumption by the microbial compartment, the abundance of which concomitantly increased over time (Fig. 4b, Table 4). The percentage of active eubacteria also evolved significantly during the experiment but to a lesser extent than the total number of bacteria (Fig. 4c). The occurrence of *N. rhenorhodanensis* did not significantly affect the microbial compartment although amphipods significantly delayed the DOC decrease during the experiment (Fig. 4a, Table 4).

A significant increase in δ^{13}C was measured in bacteria attached to sediment (Fig. 5b, two-way ANOVA, time effect, $F_{(4,18)} = 67.3$, $P < 0.001$) and tissues of amphipods (Fig. 5c, one-way ANOVA, $F_{(5,12)} = 39.9$, $P < 0.001$). The best model fit of DOC concentration (Fig. 5a) and $^{13}C/^{12}C$ ratio of total organic carbon associated with sediments (Fig. 5b) and amphipods (Fig. 5c) was obtained with parameter values reported in Table 5. According to differences in DOC decrease with time between control and fauna treatments ('time × fauna' effect in Table 4), a lower half velocity constant (K_S) and a higher maximum growth rate of bacteria (μ_1) were estimated in the control treatment in comparison with the fauna treatment (Table 5). Although amphipods delayed DOC decrease and reduced bacterial growth rate, the conversion coefficient of DOC into bacterial carbon (Y_1) was increased by 18% with *N. rhenorhodanensis*

Table 2 ANOVA results for the effects of DOC fluxes, occurrence of *N. rhenorhodanensis* and depth on removal rates of dissolved oxygen and dissolved organic carbon, microbial activities, proteins, carbohydrates and bacterial abundances in slow filtration columns (d.f.: degrees of freedom; DOC RR: dissolved organic carbon removal rate; DO RR: dissolved oxygen removal rate; INT: deshydrogenase activity; FDA: hydrolytic activity, BA: bacterial abundance; % AE: percentage of active eubacteria)

		DOC RR		DO RR		INT		FDA		Proteins		Carbohydrates		BA		% AE		Sedimentary $\delta^{13}C$	
Effect	d.f.	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*
DOC flux	2	13.20	<0.001	313.1	<0.001	11.76	<0.001	128.3	<0.001	950.3	<0.001	49.59	<0.001	226.69	<0.001	179.0	<0.001	64.69	<0.001
Fauna	1	0.25	0.62	13.12	<0.001	5.23	<0.05	0.10	0.75	0.76	0.39	0.12	0.73	0.41	0.52	0.23	0.63	0.35	0.56
Depth	2	109.5	<0.001	1884	<0.001	67.75	<0.001	0.93	0.40	84.09	<0.001	133.2	<0.001	120.54	<0.001	62.17	<0.001	185.7	<0.001
DOC flux * Fauna	2	0.28	0.75	1.08	0.35	0.53	0.59	0.85	0.43	7.08	<0.01	6.16	<0.01	0.52	0.60	0.99	0.38	0.79	0.46
DOC flux * Depth	4	11.84	<0.001	226.5	<0.001	5.68	<0.01	1.90	0.13	6.92	<0.001	1.83	0.15	2.54	0.06	1.9	0.13	32.8	<0.001
Fauna * Depth	2	0.47	0.63	5.02	<0.05	4.165	<0.05	0.56	0.58	0.32	0.73	1.63	0.21	1.54	0.23	0.9	0.41	0.43	0.65
DOC flux * Fauna * Depth	4	0.95	0.44	12.93	<0.001	1.56	0.21	0.80	0.54	1.77	0.16	3.21	<0.05	1.02	0.40	0.29	0.88	2.11	0.10
Error	36																		

(Table 5). The growth rate of amphipods reported as bacterial biomass (μ_2) was 0.35 μg C bacteria day^{-1}. The carbon conversion efficiency of bacteria biomass to amphipods (Y_2) was 0.06, indicating that *N. rhenorhodanensis* incorporated 6% of the bacterial carbon it consumed into biomass.

Discussion

In recent years, there has been increased interest in the role potentially played by fauna in ground water (Boulton, 2000; Boulton *et al.*, 2008). Several studies were performed on animal communities living in the hyporheic zone (Danielopol, 1989; Marshall & Hall, 2004; Mermillod-Blondin *et al.*, 2004) or in karstic systems (Edler & Dodds, 1996; Kinsey, Cooney & Simon, 2007; Cooney & Simon, 2009), but very few attempts were made to specifically assess the role of obligate groundwater invertebrates inhabiting alluvial aquifers. In conformance with the bottom-up hypothesis, our results clearly showed that microbial biomass and activity in our experimental columns were predominantly driven by DOC fluxes. As observed along subsurface flowpaths (Findlay *et al.*, 1993; Marmonier *et al.*, 1995; Sobczac & Findlay, 2002), sediment respiration (DO removal rate), bacterial activity and abundance were controlled by the availability of DOC flowing through the slow filtration columns. The occurrence of the subterranean amphipod *N. rhenorhodanensis* had a slight influence on microbial activities and DOC processing. For instance, even if *N. rhenorhodanensis* could increase by 97% the oxygen respiration in the uppermost 2 cm of sediments (but only with a DOC flux of 60 μg h^{-1}), an increase in DOC flux from 60 to 180 μg h^{-1} enhanced by 389% the oxygen respiration in the same sediment layer. The overriding effect of DOC supply on microbial activities (respiration rate, organic carbon consumption, dehydrogenase and hydrolytic activities), biomass (protein and carbohydrate contents) and abundances (total number of bacteria and number of active eubacteria) indicated a predominance of bottom-up control over top-down control on groundwater microbial communities in our experiments. This finding is in accordance with the generally held view that microbial growth and activities are essentially dependent on dissolved organic matter entering ground water (Ghiorse & Wilson, 1988; Goldscheider, Hunkeler & Rossi, 2006).

Table 3 Correlations between biogeochemical processes, microbial variables and sedimentary $\delta^{13}C$ obtained in slow filtration columns (DOC RR: dissolved organic carbon removal rate; DO RR: dissolved oxygen removal rate; INT: dehydrogenase activity; FDA: hydrolytic activity, BA: bacterial abundance; % AE: percentage of active eubacteria)

Variables	DOC RR	DO RR	INT	FDA	Proteins	Carbohydrates	BA	% AE
DO RR	$R = 0.912$							
	$P < 0.001$							
INT	$R = 0.786$	$R = 0.805$						
	$P < 0.001$	$P < 0.001$						
FDA	$R = 0.368$	$R = 0.425$	$R = 0.420$					
	$P < 0.05$	$P < 0.01$	$P < 0.01$					
Proteins	$R = 0.529$	$R = 0.609$	$R = 0.580$	$R = 0.873$				
	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.001$				
Carbohydrates	$R = 0.801$	$R = 0.824$	$R = 0.816$	$R = 0.463$	$R = 0.694$			
	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.01$	$P < 0.001$			
BA	$R = 0.646$	$R = 0.738$	$R = 0.717$	$R = 0.760$	$R = 0.901$	$R = 0.835$		
	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.001$		
% AE	$R = 0.565$	$R = 0.652$	$R = 0.624$	$R = 0.747$	$R = 0.897$	$R = 0.757$	$R = 0.908$	
	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.001$	
Sedimentary $\delta^{13}C$	$R = 0.866$	$R = 0.957$	$R = 0.779$	$R = 0.485$	$R = 0.630$	$R = 0.772$	$R = 0.740$	$R = 0.661$
	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.01$	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.001$

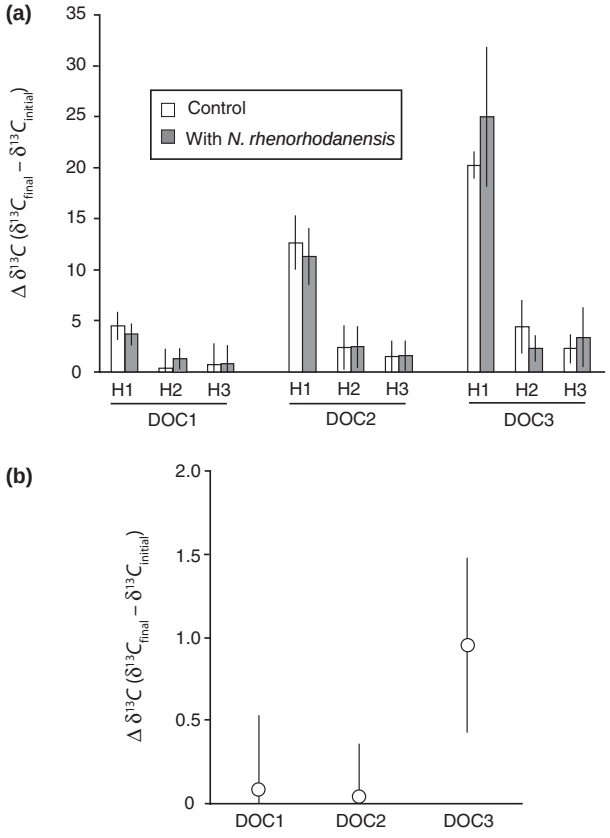

Fig. 3 Changes in $\delta^{13}C$ of total organic carbon attached to sediment (a) and tissues of *N. rhenorhodanensis* (b) during the course of the experiment I. Values are means ± SD ($n = 3$ columns per treatment).

In slow filtration columns, we detected significant increases in oxygen consumption and dehydrogenase activity of bacteria with *N. rhenorhodanensis*. With a DOC flux of 60 μg h^{-1}, amphipods produced an increase in oxygen consumption of 1.3 mg of O_2 h^{-1} L^{-1} of sediment in the uppermost 2 cm of sediments that could not be attributed to the sole respiration of 10 individuals of *N. rhenorhodanensis* (0.04 mg of O_2 h^{-1} L^{-1} of sediment calculated with respiration data of Hervant *et al.*, 1997). Such results indicate that feeding and/or bioturbation activity of *N. rhenorhodanensis* apparently had a positive influence on respiratory activity of bacteria. In batches, the occurrence of amphipods also delayed the DOC decrease (increase in *Ks* and decrease in μ_1) and increased the efficiency of DOC incorporation into bacteria (Y_1). The amphipod-induced modifications of carbon conversion efficiency Y_1 in batches and dehydrogenase activity in slow filtration columns suggested that amphipods could have slightly influenced the physiology of the microbial communities. However, this effect of *N. rhenorhodanensis* on microbial function was not associated with a significant change in the biomass (proteins and carbohydrates) and abundances of bacteria (total number and number of active bacteria). Modelling of carbon fluxes in batches clearly indicated that the feeding rate of *N. rhenorhodanensis* on sedimentary biomass was too low to influence significantly the bacterial biomass (top-down effect). The fast and efficient assimilation of DOC by

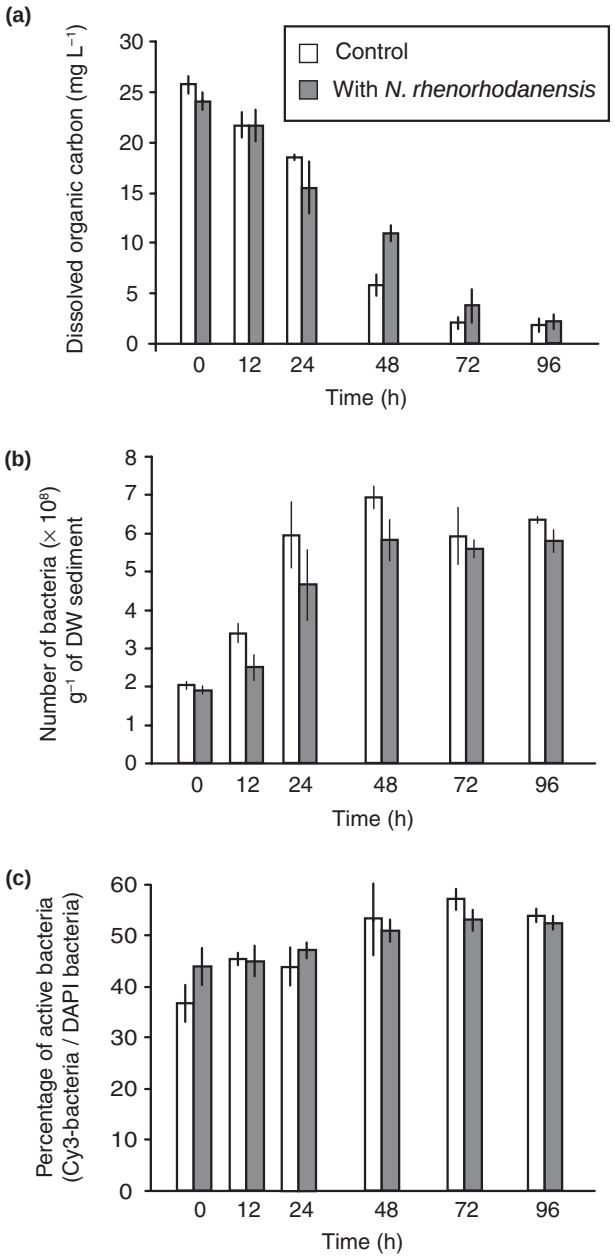

Fig. 4 Changes in the concentration of dissolved organic carbon (a), bacterial abundance (b) and percentage of active eubacteria (c) during experiment II in treatments with or without individuals of *N. rhenorhodanensis*. Values are means ± SD (n = 3 flasks per treatment).

microorganisms probably compensated for the microbial loss induced by the feeding rate of *N. rhenorhodanensis*. Indeed, we computed from our model that a total of 890 μg of DOC was incorporated into bacterial carbon in each batch during the 4 days of the experiment, whereas only 41 μg of bacterial carbon

Table 4 ANOVA results for the effects of time and occurrence of *N. rhenorhodanensis* on concentrations of dissolved organic carbon, bacterial abundances, percentages of active eubacteria and sedimentary δ^{13}C during the experiment II (d.f.: degrees of freedom for each tested effect; DOC: dissolved organic carbon; BA: bacterial abundance; % AE: percentage of active eubacteria)

		DOC concentration		BA		% AE		Sedimentary δ^{13}C	
Effect	d.f.	F	P	F	P	F	P	F	P
Fauna	1	4.13	0.18	0.36	0.58	0.49	0.53	0.09	0.78
Time	4	163	<0.001	32.9	<0.001	16.1	<0.001	67.3	<0.001
Fauna * Time	4	7.12	<0.01	2.02	0.14	1.46	0.26	0.42	0.79
Error	18								

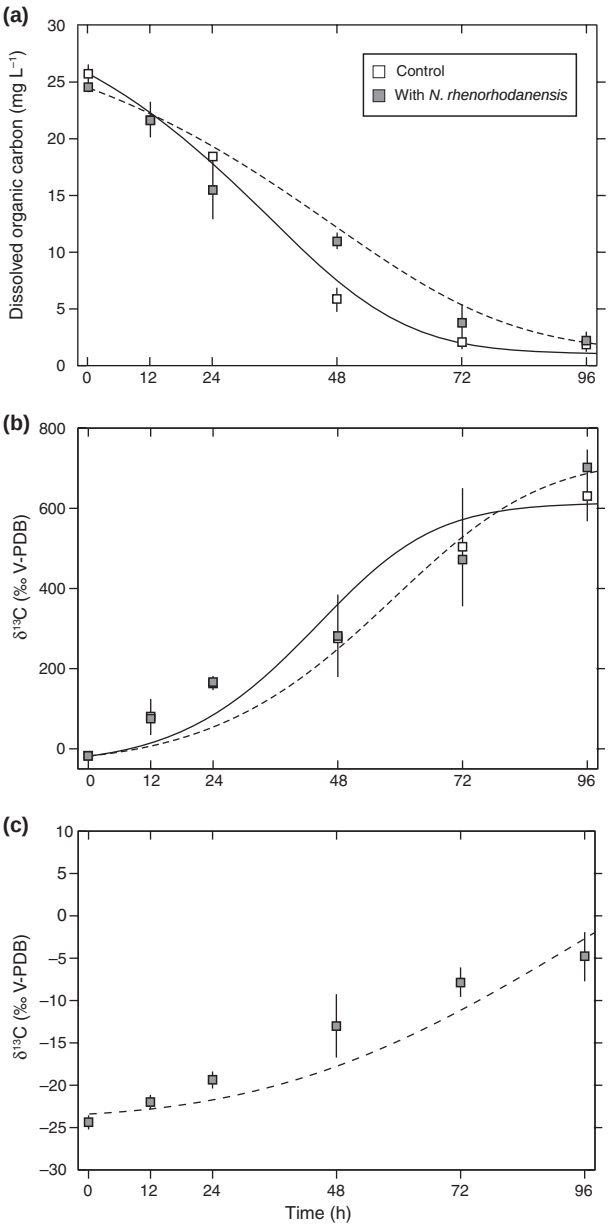

Fig. 5 Changes in DOC concentrations (a) and $\delta^{13}C$ values of total organic carbon attached to sediment (b) and *N. rhenorhodanensis* (c) during experiment II. Values are means ± SD (n = 3 flasks per treatment). Curves represent modelling results with (dashed lines) and without *N. rhenorhodanensis* (plain lines).

were fed (only 2.5 μg being incorporated in animal tissues) by amphipods during the same time period.

We did not detect any significant effect of *N. rhenorhodanensis* on the physical structure of sandy sediment. Amphipods did not influence the sediment transport coefficients (calculated with luminophore tracers), although they were observed to move in large interstices of the sediment column (visual observations on inner wall of the columns) and were collected in the whole sediment columns at the end of the experiment. The lack of sediment reworking implied that the bioturbation by amphipods did not significantly modify the living conditions for microorganisms growing on sand particles. Thus, our experiments clearly indicated that the feeding and bioturbation activities of *N. rhenorhodanensis* were much too low to significantly affect microbial processes in sediments.

Our findings differ markedly from those obtained by Edler & Dodds (1996) and Cooney & Simon (2009) with cave animals. Edler & Dodds (1996) reported a stimulation by 300–400% of attached bacteria (both abundance and activity) by the subterranean isopod *Caecidotea tridentata*, whereas organic carbon addition (D-glucose) had no effect on bacteria. They suggested that microbial activity was limited by N, rather than C, in their experiments. In such conditions, the excretion of nitrogen by isopods could have stimulated microbial abundances and activities (Edler & Dodds, 1996). In our experiments, N was not limiting because $N\text{–}NO_3^-$ concentration in water (10 mg of $N\text{–}NO_3^-$ L^{-1}) was more than tenfold higher than those used by Edler & Dodds (1996). Such comparison suggests that the influence of invertebrates on microbial processes may vary as a function of nutrient limitation. Significance of invertebrates on ecosystem processes may also depend on the functional traits of the species (Mermillod-Blondin & Rosenberg, 2006). If the amphipod *N. rhenorhodanensis* had slight effect in our experiments, Cooney & Simon (2009) reported that the cave amphipod *Gammarus minus* could reduce the bacterial production on rocks, offsetting the stimulatory effect as a result of glucose amendment. The contrasting influences of *G. minus* and *N. rhenorhodanensis* on bacterial activity were probably related to differences in metabolic activities between these two species. The ecophysiology of *N. rhenorhodanensis* and *G. minus* were studied by Hervant *et al.* (1997) and Hervant, Mathieu & Culver (1999b), respectively. At a temperature of 11 °C, the obligate groundwater amphipod *N. rhenorhodanensis* had a respiration rate of 12.9 μmol O_2 g^{-1} of dry mass h^{-1} (Hervant *et al.*, 1997). Comparatively, the amphipod *Gammarus minus* studied by Cooney & Simon (2009) exhibited a threefold higher respiration rate in normoxia at the

Table 5 Parameters used in the model and best estimated values from the fitting procedure in control and amphipod treatments

Parameters	Definition	Estimated value	
		Control	With amphipods
K_S	Monod constant (half velocity constant) (mg C)	2.55	2.66
μ_1	Maximum growth rate for bacteria (day^{-1})	1.17	1.11
μ_2	Growth rate parameter for amphipods (μg C bacteria $day)^{-1}$	–	0.35
μ_3	Rate of natural decay (day^{-1})	<0.05	<0.05
Y_1	Carbon conversion efficiency between DOC and bacteria [g C (g C bacteria)$^{-1}$]	0.22	0.26
Y_2	Carbon conversion efficiency between bacteria and *N. rhenorhodanensis* [g C bacteria (g C amphipods)$^{-1}$]	–	0.06

same temperature (between 40 and 45 μmol O_2 g^{-1} of dry mass h^{-1}, Hervant *et al.*, 1999b). Consequently, *G. minus* probably needs a threefold higher grazing activity than *N. rhenorhodanensis* to acquire its energy from microorganisms. Differences in metabolic demands could thus explain why *G. minus* can exert a higher control on microbial communities than *N. rhenorhodanensis*. In the same way, Kinsey *et al.* (2007) reported that *G. minus* produced a mean leaf litter breakdown of 0.14 g^{-1} of leaf g^{-1} of dry animal day^{-1}, whereas recent laboratory experiments showed that *Niphargus rhenorhodanensis* had a 20-fold lower effect on leaf litter breakdown (0.007 g^{-1} of leaf g^{-1} of dry animal day^{-1}, Simon Navel, unpubl. data).

The low metabolism of *N. rhenorhodanensis* is a characteristic shared by most subterranean species (Hüppop, 1985; Hervant *et al.*, 1997). Indeed, most subterranean invertebrates have developed efficient energy-saving strategies (low metabolism, storage of energy reserves) for fitting their physiological function to food-limited conditions prevailing in most aquifers (e.g. Hüppop, 1985; Danielopol *et al.*, 1994; Hervant & Renault, 2002). Hervant *et al.* (1997) also demonstrated that *N. rhenorhodanensis*, by depressing its metabolism, could survive without feeding for periods largely exceeding 200 days. We can therefore presume that the low influence of *N. rhenorhodanensis* on microorganisms in our experiments was linked to its low metabolic demand. However, we cannot exclude that the low influence of this omnivorous amphipod on microorganisms might have resulted from a low feeding preference for sedimentary bacteria. Although experiments showed that *N. rhenorhodanensis* could be maintained several years in laboratory under a large variety of foods (leaves, sediment, meat) (Ginet, 1960; Mathieu, 1967), knowledge about the feeding preferences of this species are lacking and we only detected small quantities of faecal pellets with fine sediment in boils used for gut content clearing (personal observation). Further experiments in the laboratory and in the field (using $\delta^{13}C$ and $\delta^{15}N$ to reconstitute food webs) are therefore greatly needed to determine the feeding preferences of *N. rhenorhodanensis* in the alluvial ecosystems. Nevertheless, the present study highlighted that, in sand-gravel sediments characteristics of the alluvial aquifer of the Rhône River, *N. rhenorhodanensis* was not able to influence significantly dissolved organic matter processing (DOC removal rate). This result was linked to the lack of significant activity of the amphipods in sandy zones where microbial processes predominantly occurred in our experimental systems (Mermillod-Blondin *et al.*, 2001). Although *N. rhenorhodanensis* is a dominant hypogean species of the alluvial aquifer of the Rhône River, other less abundant subterranean organisms that are strict microbivores and effective sediment bioturbators would have more influence on microbial processes than amphipods. In surface environments, tubificid worms such as *Tubifex tubifex* or *Limnodrilus hoffmeisteri* are known to strongly affect organic matter processing in freshwater sediments (Pelegri & Blackburn, 1995; Mermillod-Blondin *et al.*, 2001). Therefore, experiments with strict subterranean tubificid worms of the genus *Trichodrilus* (Creuzé des Châtelliers *et al.*, 2009) will be needed to more widely explore the potential influence of subterranean fauna on microbial processes in alluvial sediments. Moreover, comparisons of physiological traits and roles of tubificid worms living in both surface and subterranean habitats would allow us to determine whether it exits a

relationship between the ecological status of the animals, their metabolic rates and their roles in ecosystem functioning.

Finally, our laboratory experiments did not confirm the assumption of Boulton *et al.* (2008) according to which subterranean amphipods could have a significant influence on organic matter processing in interstitial systems. Microbial activity was tightly linked to the flux of dissolved organic matter in slow filtration columns, but the feeding and burrowing activities of *N. rhenorhodanensis* were far too low for influencing microbial processes. We suggest that the feeding preferences and the low metabolism of *N. rhenorhodanensis* probably induced by a natural adaptation to low-food conditions might have severely reduce its ability to influence its environment. Testing our hypothesis of a relationship between metabolism and functional role of subterranean fauna needs to extend our experiments to a wide range of strict microbivore organisms, including species with multiple populations living in habitats with contrasted food supply.

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